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16. ABSTRACT

Reproduction of rainbow trout, *Salmo gairdneri* in acidic water was studied. Adults were exposed to waters of pH 4.5, 5.0, or 5.5 during the final 6 weeks of reproductive maturation; control acidities were pH 6.5-7.1. Progeny of acid-exposed females & control males had reduced survival through 7 d of development, hatching, and yolk-sac absorption, demonstrating that oogenesis is sensitive to acidic conditions. Similar reductions in the survival of the progeny of acid-exposed males & control females indicate the sensitivity of spermatogenesis to low ambient pH. Among the progeny of unexposed adults, survival through 7 d of development & hatching was lower during rearing at pH 4.5, 5.0, or 5.5 than at pH 6.5-7.1. No eggs exposed to pH 4.5 survived to the eyed stage. Concentrations of plasma estradiol-17β, androgens, and 17α-hydroxy-20β-dihydroprogesterone in acid-exposed adult rainbow trout revealed no gross physiological abnormalities. Plasma calcium concentrations in adult females were unaffected by low pH, but were decreased in adult males exposed to pH 4.5 for 42 d. Plasma sodium concentrations fell slightly in adult fish after 7 d of exposure to pH 4.5,5.0, or 5.5 and continued to decline in fish exposed to pH 4.5. We conclude that reproduction of rainbow trout is likely to be affected by environmental acidification to pH values below 5.5, although responses may be modified by other biological and physical conditions.

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Effects of Low pH on Reproduction of Rainbow Trout[1]

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Abstract.—Reproduction of rainbow trout *Salmo gairdneri* in acidic water was studied. Adults were exposed to waters of pH 4.5, 5.0, or 5.5 during the final 6 weeks of reproductive maturation; control acidities were pH 6.5–7.1. Progeny of acid-exposed females and control males had reduced survival through 7 d of development, hatching, and yolk-sac absorption, demonstrating that oogenesis is sensitive to acidic conditions. Similar reductions in the survival of the progeny of acid-exposed males and control females indicate the sensitivity of spermatogenesis to low ambient pH. Among the progeny of unexposed adults, survival through 7 d of development and hatching was lower during rearing at pH 4.5, 5.0, or 5.5 than at pH 6.5–7.1. No eggs exposed to pH 4.5 survived to the eyed stage. Concentrations of plasma estradiol-17β, androgens, and 17α-hydroxy-20β-dihydroprogesterone in acid-exposed adult rainbow trout revealed no gross physiological abnormalities. Plasma calcium concentrations in adult females were unaffected by low pH, but were decreased in adult males exposed to pH 4.5 for 42 d. Plasma sodium concentrations fell slightly in adult fish after 7 d of exposure to pH 4.5, 5.0, or 5.5 and continued to decline in fish exposed to pH 4.5. We conclude that reproduction of rainbow trout is likely to be affected by environmental acidification to pH values below 5.5, although responses may be modified by other biological and physical conditions.

Surface water acidification is a threat to fishery resources in northern Europe (Almer et al. 1974; Leivestad et al. 1976; Wright et al. 1980) and eastern North America (Haines 1981). Much concern over the impacts of acidification in these regions has focused on salmonid populations because of their commercial and recreational importance.

Reproductive failure is a major contributor to the gradual extinction of fish populations in acidic waters (Jensen and Snekvik 1972; Beamish et al. 1975; Beamish 1976; Ryan and Harvey 1977, 1980). Such failure has been attributed to physiological disruptions during gametogenesis (Beamish et al. 1975; Lockhart and Lutz 1977) and mortality of early life history stages (Jensen and Snekvik 1972; Leivestad et al. 1976). Laboratory exposures of adult female fish to acidic conditions during reproductive maturation demonstrated the potential for reductions in egg quality and quantity (Craig and Baksi 1977; Ruby et al. 1977; Lee and Gerking 1980). Survival bioassays have established that embryos and larvae are more sensitive to acidity than juveniles and adult fish (Menendez 1976; Daye and Garside 1977; Craig and Baksi 1977; Carrick 1979).

The goal of our research was to increase understanding of current and potential consequences of low environmental pH for the reproduction of salmonid fishes. This goal was addressed by studying the effects of low pH on final reproductive maturation and survival of progeny of rainbow trout *Salmo gairdneri*, a relatively acid-sensitive species among the salmonids (Grande et al. 1978). Plasma concentrations of sodium, calcium, and sex hormones were evaluated as physiological indicators of reproductive stress in acid-exposed adult fish.

Methods

Adult rainbow trout (age 2), averaging 57.4 ± 3.2 cm (mean ± SD) in total length and 2.2 ± 0.5 kg in weight, were obtained in June 1982 from the domestic broodstock of the Roaring River Hatchery (Oregon Department of Fish and Wildlife, Scio, Oregon) and transported to the Western Fish Toxicology Laboratory of the U.S. Environmental Protection Agency, Corvallis, Oregon. Blood samples were obtained (prior to October 5, 1982) by caudal puncture with a needle and ammonium-heparinized syringe, and gender of the fish was determined by the Ouchterlony Immunodiffusion Assay Technique through which vitellogenin is specifically detected in the plasma of female fish (Terry Owen, Helix Biotech, Limited, Richmond, British Columbia). On October 27, 1982, three

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females and four males were transferred into each of eight 757-L circular fiberglass tanks. The tanks were set up outdoors to provide a natural photoperiod. Fish were fed Silver Cup dry trout pellets (Murray Elevator Company, Murray, Utah) daily to satiation until spawning.

The inflowing well water was gradually softened by reverse osmosis (Purification Techniques, Incorporated, Brielle, New Jersey) from 23 mg/L as $CaCO_3$ to 10 mg/L over a 5-d period. After fish acclimated to this water for an additional 3 d, reagent grade sulfuric acid was introduced into the system over 5 d until pH values of 4.5, 5.0, and 5.5 were reached in duplicate treatment tanks; softened water (pH range 6.5–7.1) was delivered to the two control tanks.

Total water hardness, determined at least twice daily by the EDTA titrimetric method (APHA et al. 1980), was 9.2 ± 1.2 mg/L (mean ± SD) as $CaCO_3$ during the experiment. A stock solution of softened water was acidified with sulfuric acid and maintained at pH 4.0 (range 3.9–4.1) with a Beckman Model 942 pH Monitor (Beckman Instruments, Incorporated, Fullerton, California). The acidified stock solution was vigorously aerated to remove carbon dioxide that may have been generated by the addition of acid, and pumped to the head tank of a gravity-flow diluter. The acidic stock solution was diluted with softened aerated well water to attain test pH values and delivered at a rate of 4.5 L/min to the tanks in the flow-through system. Treatment acidities were automatically monitored and recorded every 3 h and the diluter was manually adjusted as necessary in order to maintain exposure levels within ±0.1 pH unit of 4.5, 5.0, and 5.5. Water temperatures throughout the experiment fluctuated between 10.9 and 14.0°C without a discernible pattern. Alkalinity, determined by Gran titration as described by Stumm and Morgan (1981), was 252 ± 11 microequivalents per liter (mean ± SD) prior to addition of acid. Chemical concentrations in the softened and acidified waters did not exceed mean values reported for the toxicology station by Chapman (1978).

Blood samples were drawn from the caudal artery of each anesthetized fish with a needle and ammonium-heparinized syringe and the plasma was stored at −20°C. Anesthetic solutions (tricaine, 50 mg/L) were titrated to treatment pH values with 0.1 M NaOH to minimize stress. The first blood sample was taken after the fish were acclimated to the tanks, just before acid was added to the system. The second sample was taken 7 d after treatment acidities were reached, and the final sample was drawn at the time of spawning.

Concentrations of plasma estradiol-17β, androgen (testosterone and 11-ketotestosterone), and 17α-hydroxy-20β-dihydroprogesterone were determined by radioimmunoassay by the general method of Korenman et al. (1974). Details of the procedure were described by Sower and Schreck (1982). Estradiol-17β was determined in 10 µL of plasma for males and 25 µL of plasma for females. Androgens were determined in 10 µL of plasma and 17α-hydroxy-20β-dihydroprogesterone was determined in 25 µL of plasma. Samples were extracted twice with diethyl ether and extraction efficiency was about 90% for all assays. Antiestradiol-17β and antitestosterone were provided by G. Niswender (Colorado State University, Fort Collins, Colorado) and anti-17α-hydroxy-20β-dihydroprogesterone was provided by A. P. Scott (Ministry of Agriculture and Fisheries, Lowestoft, Suffolk, United Kingdom). Antibody characteristics were given by Sower and Schreck (1982) and Scott et al. (1982). Plasma calcium concentrations were determined by atomic absorption flame emission spectrophotometry, and sodium concentrations were measured by flame photometry.

Fish were examined for reproductive maturity weekly, beginning with the fourth week of exposure. After 42 d of exposure (December 21, 1982), the ripe fish were anesthetized for blood sampling, killed with a blow to the head, and manually spawned. The gametes were collected separately from each fish; gametes from control fish were pooled before fertilization and those from acid-exposed fish were held separately for fertilization and rearing. Dry fertilizations, followed by 1 h of water hardening, provided progeny from four crosses.

(A) *Control males* × *control females.* Water hardening and rearing took place at each of the acidities used in the prespawning exposure, to enable us to assess the performance at low pH of the progeny of control adults.

(B) *Control males* × *acid-exposed females.* Eggs from females exposed to each acidity were fertilized with sperm from control males. Water hardening and rearing took place at the control pH to determine the effects of acidic water on oogenesis.

(C) *Acid-exposed males* × *control females.* Eggs from control females were fertilized with sperm from males from each acidity. Water hardening and rearing took place at the con-

TABLE 1.—Survival of progeny from acid-exposed and control rainbow trout. Numbers of males and females that contributed to each group are shown in parentheses.

Parents, prespawning pH		Progeny		Eggs, % viability	% survival first 7 d (*N*)	% eyed eggs hatched (*N*)	% larvae surviving to yolk-sac absorption (*N*)	Cumulative % survival[a]
Males	Females	Group	Rearing pH					
Cross A: control males × control females								
Control (7)	Control (5)	A-1	Control	95	91.3 (689)	93.8 (465)	86.2 (436)	73.8
Control (7)	Control (5)	A-2	5.5	98	[b]64.2 (749)	[b]89.2 (381)	92.4 (340)	52.9
Control (7)	Control (5)	A-3	5.0	96	[b]54.7 (766)	[b]80.1 (294)	81.5 (238)	35.7
Control (7)	Control (5)	A-4	4.5	95	[b]0 (761)			0
Cross B: control males × acid-exposed females								
Control (7)	5.5 (4)	B-1	Control	94	91.9 (211)	[b]57.8 (154)	[b]70.8 (89)	37.6
Control (7)	5.0 (6)	B-2	Control	97	[b]99.1 (439)	91.1 (426)	88.9 (388)	80.3
Control (7)	4.5 (4)	B-3	Control	95	93.1 (189)	[b]26.4 (148)	89.7 (39)	22.0
Cross C: acid-exposed males × control females								
5.5 (8)	Control (5)	C-1	Control	99	93.8 (689)	[b]80.8 (386)	[b]79.5 (312)	60.2
5.0 (8)	Control (5)	C-2	Control	99	93.2 (883)	[b]75.0 (641)	[b]82.7 (481)	57.8
4.5 (7)	Control (5)	C-3	Control	98	[b]87.4 (933)	[b]58.6 (534)	86.6 (313)	44.4
Cross D: acid-exposed males × acid-exposed females								
5.5 (8)	5.5 (4)	D-1	5.5	94	60.3 (136)	[b]57.8 (71)	[c]82.9 (41)	28.9
5.0 (8)	5.0 (6)	D-2	5.0	94	[d]76.8 (555)	84.0 (374)	77.7 (314)	50.1
4.5 (7)	4.5 (4)	D-3	4.5	96	0 (244)			0

[a] Product of rates in the three preceding columns.
[b] Different from group A-1 at the same development stage: χ^2 test; $P < 0.05$.
[c] Different from group A-2 at the same development stage: χ^2 test; $P < 0.05$.
[d] Different from group A-3 at the same development stage: χ^2 test; $P < 0.05$.

trol pH to determine the effects of acidic water on spermatogenesis.

(D) *Acid-exposed males × acid-exposed females.* Eggs from females exposed to each acidity were fertilized with sperm from males exposed to the same acidity. Water hardening and rearing took place at the respective parental acidities to assess reproductive performances when both parents and the progeny were exposed to low pH.

Aliquots of embryos from each fertilization group were placed in incubation cells within drawers of Heath incubators for rearing. The cells consisted of 4.6-cm-long sections of 6.4-cm-diameter polyvinyl chloride pipe with plastic mesh screen attached across one end, forming a cup. Similar cups, shorter but wider, were inverted over each cell to confine the fish after they hatched. The flow rate through the incubators was 2 L/min. The incubators were covered with black plastic sheets to exclude light. Dead eggs and larvae were counted and removed daily (except as noted later). A different incubator was used for each test pH (4.5, 5.0, 5.5, and control). Within a treatment, cells of eggs were randomly distributed throughout the incubator.

Some eggs became infected with *Saprolegnia* fungi on the eighth day of development. Consequently, we tallied the mortalities through the last day before the occurrence of infection (after 7 d of development), removed the infected eggs, and monitored the remaining eyed eggs for survival through hatching. Survival through yolk-sac absorption was then determined among the hatched fish. Progeny survival rates were statistically analyzed with chi-square contingency tables (Cochran and Cox 1957). Survival rates were first statistically tested within each mating group. Because no individual differences were found, survival data were pooled for comparisons among treatments. Plasma calcium and sodium concentrations were evaluated by analysis of variance and by Duncan's new multiple-range test for comparisons among means (Steel and Torrie 1980).

Results

Survival Rates

Survival through 7 d and hatching success were lower for progeny of control fish that were reared at pH 5.5 and 5.0 (groups A-2 and A-3) than for those reared in control water (group A-1). No embryos reared at pH 4.5 (groups A-4 and D-3) survived through 7 d (Table 1).

Influences of acid exposure on oogenesis were

indicated by the reduced hatching percentages among the progeny of females that had been exposed to pH 5.5 or 4.5 (groups B-1 and B-3). Survival from hatching through yolk-sac absorption was lower for progeny of females that had been exposed to pH 5.5 (group B-1) than for the controls (group A-1).

Impaired spermatogenesis was evident in the performances of the progeny of acid-exposed males (groups C-1, C-2, and C-3). Survival through 7 d of development and hatching success were lower among progeny of males exposed to pH 4.5 before spawning (group C-3) than among the progeny of control males (group A-1); and the survival through hatching and yolk-sac absorption was lower for the progeny of males exposed to pH 5.5 or 5.0 (groups C-1 and C-2) than for the progeny of control fish (group A-1).

Hatching success and survival through yolk-sac absorption were lower when both parents and the progeny were exposed to pH 5.5 (group D-1) than when only the progeny were exposed to that pH (group A-2). Females exposed to pH 5.0 produced gametes of high quality, as reflected by the survival of their progeny in control water and at pH 5.0 (groups B-2 and D-2) through 7 d of development, in comparison with the performances of the progeny of control females (groups A-1 and A-3).

Plasma Constituents

No clear indications of acid stress were apparent in the plasma calcium concentrations of females (Table 2). Plasma calcium decreased in males that were exposed to pH 4.5 for 42 d ($P < 0.05$). Plasma sodium concentrations in the adults tended to decrease after 7 d of exposure to each treatment pH. In fish exposed to pH 4.5 for 42 d, sodium concentrations were sharply reduced in comparison with those of control fish ($P < 0.05$). Concentrations of estradiol-17β, androgens, and 17α-hydroxy-20β-dihydroprogesterone in adult fish did not differ among treatments. There was no apparent effect of acid exposure on rate of sexual development; except for one control female and two females exposed to pH 5.5, the fish ovulated at the same time. The three exceptions ovulated less than 1 week after the matings were conducted but their gametes were excluded from the experiment because the use of different sperm batches for fertilization would have confounded interpretation of the results. When pressure was applied to the abdomen, eggs from all of the females flowed easily from the ovipore. No eggs appeared to be atretic. In two of the females held at pH 4.5, approximately 10% of the eggs in the ovaries had not ovulated and were held tightly in the skein, although the remainder of the eggs were fully mature and ovulated. All males were ripe, having copious quantities of sperm at the time of spawning. Samples of sperm from each male were observed with a dissecting microscope and appeared equally motile.

Hatching occurred synchronously among embryos reared in unacidified water and at pH levels of 5.5 and 5.0. Two adult females died at pH 4.5 (after 29 and 34 d of exposure) as did one male (after 35 d). One male died in control water after 11 d.

Discussion

We have shown that exposures of adult rainbow trout during final reproductive maturation and direct exposures of embryos and larvae to waters of pH 5.5. and below may affect reproductive success. Both oogenesis and spermatogenesis were impaired. Effects of acid exposure on gametogenesis have previously been demonstrated histologically and through determinations of egg production and fertility in flagfish *Jordanella floridae* at pH 6.0 and lower (Craig and Baksi 1977). Similarly, Lee and Gerking (1980) observed reductions in egg quality and quantity in desert pupfish *Cyprinodon nevadensis* below pH 7.0. Examination of the cumulative percent survival of the offspring of acid-exposed fish suggested that oogenesis was affected more seriously than was spermatogenesis at pH 5.5 or 4.5 in rainbow trout. The enhanced survival through 7 d of development of progeny of females exposed to pH 5.0 before spawning remains unexplained.

Direct effects of acidity on early development were shown by the increased mortality at pH 5.5, 5.0, and 4.5 of the progeny of control fish. The first 7 d of development and hatching were the stages most sensitive to the acid, and these periods have been identified as sensitive to toxicants in general (Rosenthal and Alderdice 1976).

Disruptions of plasma calcium regulation associated with vitellogenesis have been implicated as a contributing factor to reproductive failure in acid-stressed fish populations (Beamish et al. 1975; Lockhart and Lutz 1977). Plasma calcium concentration is known to increase in conjunction with vitellogenesis in salmonids (Bromage et al. 1982); the concentration then decreases gradually during final reproductive maturation (Whitehead et al. 1978). Plasma calcium concentrations in our acid-

TABLE 2.—Plasma concentrations of calcium, sodium, and sex hormones for adult control and acid-exposed rainbow trout; meq = milliequivalents.

Sex and exposure pH	Before acidification (57 d prespawning)			7 d acid exposure (35 d prespawning)			42 d acid exposure (spawning)		
	N	Mean	SE or range	*N*	Mean	SE or range	*N*	Mean	SE or range
Calcium (meq/L)									
Females									
Control	6	8.2	0.5	6	8.7	0.7	5	7.3	1.6
5.5	6	5.8	0.6	6	6.3	0.6	4	5.7	0.8
5.0	6	6.4	0.5	6	7.0	0.4	6	7.5	1.5
4.5	6	7.6	0.7	6	6.2	0.5	4	5.5	0.8
Males									
Control	8	5.3	0.2	7	5.8	0.2	7	5.2	0.1
5.5	7	5.2	0.1	8	5.2	0.2	6	5.8	0.3
5.0	7	5.4	0.1	8	5.5	0.2	8	5.3	0.1
4.5	8	5.5	0.2	8	5.5	0.3	7	4.8[a]	0.1
Sodium (meq/L)									
Both sexes[b]									
Control	14	153	1	14	152	1	12	154	2
5.5	13	152	3	14	143	4	10	143	6
5.0	13	153	1	14	146	3	14	147	4
4.5	14	153	2	13	145	4	11	129[a]	4
Estradiol-17β (ng/mL)									
Females									
Control	6	2.9	1.2–5.6	6	3.4	1.3–5.8	5	0.6	<0.1–1.3
5.5	6	3.4	1.4–7.6	6	3.7	2.4–5.5	4	0.3	0.1–0.5
5.0	6	3.0	1.0–5.5	6	4.0	1.7–5.4	6	0.7	0.3–0.8
4.5	6	2.9	1.1–4.1	6	3.3	1.1–4.8	4	0.7	<0.1–1.5
Males									
Control	8	0.1	<0.1–0.2	7	<0.1	<0.1–0.1	7	<0.1	<0.1–0.1
5.5	7	0.1	<0.1–0.2	8	<0.1	<0.1–0.1	6	<0.1	<0.1–0.1
5.0	7	0.1	<0.1–0.3	8	0.1	<0.1–0.3	8	0.1	<0.1–0.1
4.5	8	0.1	<0.1–0.2	8	0.1	<0.1–0.2	7	0.1	<0.1–0.1
Androgens (ng/mL)									
Females									
Control	6	23	15–31	6	26	13–48	5	16	13–18
5.5	6	18	14–24	6	25	15–42	4	18	13–23
5.0	6	19	13–23	6	26	10–67	6	18	11–29
4.5	6	21	16–26	6	27	20–33	4	14	13–15
Males									
Control	8	29	17–48	7	22	13–35	7	21	13–46
5.5	7	24	21–27	8	31	17–67	6	27	15–35
5.0	7	33	16–59	8	19	17–25	8	21	10–34
4.5	8	26	18–37	8	31	19–64	7	33	14–61
17α-hydroxy-20β-dihydroprogesterone (ng/mL)									
Females									
Control	6	0.2	<0.1–0.7	6	0.6	<0.1–2.9	5	24.6	15.3–33.9
5.5	6	0.1	<0.1–0.7	6	0.7	<0.1–2.4	4	32.1	21.1–43.1
5.0	6	<0.1	<0.1–0.3	6	0.6	<0.1–1.0	6	26.7	6.8–50.9
4.5	6	<0.1	all <0.1	6	1.2	0.4–5.8	4	37.0	36.2–39.5

[a] Different from the mean value in the same exposure group prior to acidification and the mean value in the control group at spawning; Duncan's new multiple-range test; $P \leq 0.05$.

[b] Mean concentrations for females and males were compared by a *t*-test; no significant differences were found; thus data were pooled for comparisons among treatments.

exposed female rainbow trout tended to decrease as expected but appeared to do so independently of pH level. Perhaps acid exposure over a greater portion of the vitellogenic process would result in discernible plasma calcium dysfunction. Indeed, plasma calcium concentrations in male rainbow trout tend to be relatively invariant, but they were reduced after exposure to pH 4.5 for 42 d.

The low plasma sodium concentrations in adult rainbow trout exposed to pH 4.5 for 6 weeks were consistent with current theory on the effects of acidic conditions on ionoregulation. Packer and Dunson (1970) showed that low pH conditions decrease sodium influx rates and increase efflux rates. Ambient acidity is believed to increase the permeability of the gill epithelium to sodium, accounting for the increased efflux rates (McWilliams and Potts 1978). McDonald et al. (1980) determined that ionoregulation and acid–base balance in acid-exposed rainbow trout are influenced by water hardness. Acute acid exposure in soft water produces severe ionoregulatory dysfunction, but only minor acidosis. In hard water, ionoregulation is slightly affected and profound acidosis may occur. Sublethal effects on sodium regulation of adult rainbow trout at pH 4.5 may have little meaning in the reproductive process, because no embryos survived to the eyed stage at this pH.

Sex hormone concentrations revealed no gross physiological abnormalities attributable to acid exposure. Hormone profiles in the acid-exposed fish followed patterns typical for this species (Scott and Sumpter 1983). These hormone concentrations are dynamically regulated and may vary widely among individuals (Schreck et al. 1972; Hille 1982); therefore, they may be unsuitable as indicators of reproductive stress at sublethal pH. However, low male sex hormone concentrations were found in the plasma of adult male Atlantic salmon *Salmo salar* in river water of about pH 4.7 (Freeman et al. 1983).

Our data suggest that the reproductive capacity of rainbow trout will be reduced in waters of pH 5.5 or lower. The effects of acidity upon the recruitment of rainbow trout may be more severe than our data suggest, however, because we addressed only a limited portion of the reproductive cycle, and the most critical life history stages may not have been identified. Further studies should consider the effects of acid exposure earlier in the maturation process as well as the sensitivity of juvenile rainbow trout at first feeding. In addition, recruitment success may be influenced by such water quality characteristics as hardness, temperature, or the presence of other toxicants, primarily metals (see review by Spry et al. 1981), and biological factors. Low pH has been known to inhibit spawning behavior of brook trout *Salvelinus fontinalis* (Johnson and Webster 1977) and to decrease sperm motility in northern pike *Esox lucius* (Duplinsky 1982). We noted that rainbow trout yolk-sac larvae were lethargic at pH levels of 5.5 and 5.0; similar behavior was noted for buttoned-up pink salmon *Oncorhynchus gorbuscha* and chum salmon *Oncorhynchus keta* (Rombough 1983). The reduction in general activity may be important insofar as it impinges on behaviors like the rotation of pectoral fins, which generate currents for the renewal of oxygen supply in gravel interstices, or in the avoidance of predators.

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